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<p>(54) Title: HEPATITIS-C VIRUS TYPE 4, 5 AND 6 (57) Abstract  Newly elucidated sequences of hepatitis C virus type 4 and type 5 are described, together with those of a newly discovered type 6. Unique type-specific sequences in the NS4, NS5 and core regions enable HCV detection and genotyping into types 1 to 6. Antigenic peptides and immunoassays are described.</p>		

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HEPATITIS-C VIRUS TYPE 4, 5 and 6Technical Field

The present invention relates to newly elucidated sequences of hepatitis C virus type 4 (HCV-4), and type 5 (HCV-5), and to a newly discovered type 6 (HCV-6). In particular, it relates to the etiologic agent of hepatitis C virus type 4, 5 and 6, and to polynucleotides and immunoreactive polypeptides which are useful in immunoassays for the detection of HCV-4, HCV-5 and HCV-6 in biological samples; and also to the use of antigenic HCV-4, HCV-5 and HCV-6 specific polypeptides in vaccines.

Background of the Invention

Acute viral hepatitis is a disease which may result in chronic liver damage. It is clinically diagnosed by a well-defined set of patient symptoms, including jaundice, hepatic tenderness, and an increase in the serum levels of alanine aminotransferase and aspartate aminotransferase. Serologic immunoassays are generally performed to diagnose the specific type of viral causative agent. Historically, patients presenting with symptoms of hepatitis and not otherwise infected by hepatitis A, hepatitis B, Epstein-Barr or cytomegalovirus were clinically diagnosed as having non-A, non-B hepatitis (NANBH) by default.

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The HCV genome encodes a large polyprotein precursor, which contains structural and non-structural regions. The single protein is apparently cleaved into a variety of proteins after production. Most of the structural and non-structural proteins have now been identified from in vitro RNA translation and expression as recombinant proteins. The C and E regions encode for nucleocapsid structural proteins and for envelope structural proteins, respectively. At least five additional regions follow, which encode for non-structural (NS) protein of undefined function. The organisation is believed to be as follows (A. Alberti, Journal of Hepatology, 1991; 12; 279 to 282)

5' 3'  
NCR: C : E1 : E2 : NS1 : NS2 : NS3 : NS4 : NS5

Certain immunoreactive proteins have been described as recombinant proteins, for example C22 (in the core region), C33 (in NS3 region), 5-1-1 and C100 (both in the NS4 region), and NS5 (NS5 region). Current diagnosis of hepatitis C is often based on methods which detect antibodies against the product of the C-100 clone. This

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clone was produced by ligation of overlapping clones to produce a larger viral antigen (C100) corresponding to part of the NS3-NS4 genomic region. C100 was then fused with the human superoxide dismutase (SOD) gene, expressed in use as a large recombinant fusion protein (C100-3) and used on solid phase to develop radio-labelled (RIA) and enzyme-linked immunosorbent assays (ELISA).

Polynucleotides alleged to be useful for screening for HCV are disclosed in European Patent Specification EP-A-0398748. European Patent Specification EP-A-0414475 purports to disclose the propagation of HCV in culture cells and the production of antigens for use in diagnostics. European Patent Specification EP-A-0445423 discloses what is stated to be an improved immunoassay for detecting HCV antibodies.

Blood banks in the United Kingdom carry out routine testing of blood donors for antibodies to components of HCV. A first generation assay involved the detection of HCV antibodies to C100-3 polypeptides. The C100-3 antibody recognises a composite polyprotein antigen within non-structural regions of the virus and is a consistent marker of HCV infection. However, in acute infections this antibody is unreliable because of the delay (typically 22 weeks) in seroconversion after exposure. Furthermore, the C100-3 antibody test lacks specificity for the hepatitis C virus.

Second generation antibody tests employ recombinant

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antigens and/or synthetic linear peptides representing structural antigens from the highly conserved core region of the virus as well as non-structural antigens. However, it is found that some second-generation ELISA tests can yield false-positive reactions. The recombinant immunoblot assay (RIBA-2) incorporating four antigens from the HCV genome, purports to provide a method for identifying genuine-anti-HCV reactivity. However, the result can be "indeterminate". The present workers have reported (The Lancet, 338; Oct.19, 1991) varying reactivity of HCV-positive blood donors to 5-1-1, C100, C33 and C22 antigens, and compared these with the results of the direct detection of HCV RNA present in the blood samples using polymerase chain reaction (PCR) to amplify HCV polynucleotides. However, the work demonstrates that the unambiguous diagnosis of HCV infections is not yet possible.

Recently there has been discovered further types of HCV that differ considerably in sequence and these have been called HCV-2, 3 and 4. Our patent application WO93/10239 (published on 27th May, 1993) describes certain antigenic sequences of HCV-2, 3 and 4. The sequences disclosed for HCV-4 are in the 5' NCR and core regions only. The former does not code for any protein which might be used in an immunoassay for HCV whilst the core region tends to be conserved.

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Summary of the Invention

The present invention includes the discovery of a previously unknown type 6 variant of HCV, by a comparison of sequences amplified by polymerase chain reaction (PCR) in certain regions of the HCV genome and confirmed by phylogenetic analysis. The invention has identified polynucleotide sequences and polypeptides which are HCV-4, HCV-5 and HCV-6 specific. These may be used to diagnose HCV-4, HCV-5 and HCV-6 infection and should thus be included in any definitive test for HCV infection.

One aspect of the present invention provides a polynucleotide having a nucleotide sequence unique to hepatitis virus type 4, 5 or 6.

The sequences are unique to the HCV type concerned in the sense that the sequence is not shared by any other HCV type, and can thus be used to uniquely detect that HCV-type. Sequence variability between HCV 4, 5 and 6 has been found particularly in the NS4, NS5 and core regions and it is therefore from these regions in particular that type-specific polynucleotides and peptides may be obtained. The term type-specific indicates that a sequence unique to that HCV type is involved. Moreover, within each HCV type a number of sub-types may exist having minor sequence variations.

The invention includes NS5 polynucleotide sequences

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unique to hepatitis C virus types 4 and 6 (HCV-4 and HCV-6); and NS4 sequences unique to HCV-4, HCV-5 and HCV-6 respectively. The sequences may be RNA or DNA sequences, including cDNA sequences. If necessary the DNA sequences may be amplified by polymerase chain reaction. DNA sequences can be used as a hybridisation probe. The sequences may be recombinant (i.e. expressed in transformed cells) or synthetic and may be comprised within longer sequences if necessary. Equally, deletions, insertions or substitutions may also be tolerated if the polynucleotide may still function as a specific probe. Polynucleotide sequences which code for antigenic proteins are also particularly useful.

Another aspect of the invention provides a peptide having an amino acid sequence unique to hepatitis virus type 4, 5 or 6.

The invention includes antigenic HCV-4 or HCV-6 specific polypeptide from the NS5 region, or antigenic HCV-4, HCV-5 or HCV-6 specific polypeptide from the NS4 region; or polypeptides including these antigens. A plurality of copies of the peptide may be bound to a multiple antigen peptide core.

The peptide may be labelled to facilitate detection, and may for example be labelled antigenic HCV-4 or HCV-6 specific polypeptide from the NS5 region, or labelled antigenic HCV-4, HCV-5 or HCV-6 specific polypeptide from the NS4 region; (or mixtures thereof) for use in an



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immunoassay to detect the corresponding antibodies.

It should be understood that the polypeptides will not necessarily comprise the entire NS4 or NS5 region, but that characteristic parts thereof (usually characteristic epitopes) unique to a particular type of HCV may also be employed.

A further aspect of the invention provides antibodies to the peptides, especially to HCV-4 or HCV-6 NS5 antigens, or to HCV-4, HCV-5 or HCV-6 NS4 antigens, particularly monoclonal antibodies for use in therapy and diagnosis. Thus labelled antibodies may be used for in vivo diagnosis. Antibodies carrying cytotoxic agents may be used to attack HCV-4, HCV-5 or HCV-6 infected cells.

A further aspect of the invention provides a vaccine comprising immunogenic peptide, especially HCV-4 or HCV-6 NS5 polypeptide, or immunogenic HCV-4, HCV-5 or HCV-6 NS4 polypeptide.

A further aspect of the invention provides a method of in vitro HCV typing which comprises carrying out endonuclease digestion of an HCV-containing sample to provide restriction fragments, the restriction pattern being characteristic of HCV-4, HCV-5 or HCV-6.

Finally, the present invention also encompasses assay kits including polypeptides which contain at least one epitope of HCV-4, HCV-5 or HCV-6 antigen (or antibodies thereto), as well as necessary preparative reagents, washing reagents, detection reagents and signal producing reagents.

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The HCV-4, HCV-5 or HCV-6 specific polynucleotide sequences may be used for identification of the HCV virus itself (usually amplified by PCR) by hybridisation techniques.

Oligonucleotides corresponding to variable regions, e.g. in the NS5 or NS4 region, could be used for type-specific PCR. Outer sense and inner sense primers may be used in combination with the two conserved anti-sense primers for a specific detection method for HCV types 4, 5 and 6.

The present invention also provides expression vectors containing the DNA sequences as herein defined, which vectors being capable, in an appropriate host, of expressing the DNA sequence to produce the peptides as defined herein.

The expression vector normally contains control elements of DNA that effect expression of the DNA sequence in an appropriate host. These elements may vary according to the host but usually include a promoter, ribosome binding site, translational start and stop sites, and a transcriptional termination site. Examples of such vectors include plasmids and viruses. Expression vectors of the present invention encompass both extrachromosomal vectors and vectors that are integrated into the host cell's chromosome. For use in E.coli, the expression vector may contain the DNA sequence of the present invention optionally as a fusion linked to either the 5'-

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or 3'- end of the DNA sequence encoding, for example, B-galactosidase or the 3'- end of the DNA sequence encoding, for example, the trp E gene. For use in the insect baculovirus (AcNPV) system, the DNA sequence is optionally fused to the polyhedrin coding sequence.

The present invention also provides a host cell transformed with expression vectors as herein defined.

Examples of host cells of use with the present invention include prokaryotic and eukaryotic cells, such as bacterial, yeast, mammalian and insect cells. Particular examples of such cells are E.coli, S.cerevisiae, P.pastoris, Chinese hamster ovary and mouse cells, and Spodoptera frugiperda and Tricoplusia ni. The choice of host cell may depend on a number of factors but, if post-translational modification of the HCV viral peptide is important, then an eukaryotic host would be preferred.

The present invention also provides a process for preparing a peptide as defined herein which comprises isolating the DNA sequence, as herein defined, from the HCV genome, or synthesising DNA sequence encoding the peptides as defined herein, or generating a DNA sequence encoding the peptide, inserting the DNA sequence into an expression vector such that it is capable, in an appropriate host, of being expressed, transforming host cells with the expression vector, culturing the transformed host cells, and isolating the peptide.

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The DNA sequence encoding the peptide may be synthesised using standard procedures (Gait, Oligonucleotide Synthesis: A Practical Approach, 1984, Oxford, IRL Press).

The desired DNA sequence obtained as described above may be inserted into an expression vector using known and standard techniques. The expression vector is normally cut using restriction enzymes and the DNA sequence inserted using blunt-end or staggered-end ligation. The cut is usually made at a restriction site in a convenient position in the expression vector such that, once inserted, the DNA sequences are under the control of the functional elements of DNA that effect its expression.

Transformation of an host cell may be carried out using standard techniques. Some phenotypic marker is usually employed to distinguish between the transformants that have successfully taken up the expression vector and those that have not. Culturing of the transformed host cell and isolation of the peptide as required may also be carried out using standard techniques.

The peptides of the present invention may thus be prepared by recombinant DNA technology, or may be synthesized, for example by using an automatic synthesizer.

The term "peptide" (and "polypeptide") is used herein to include epitopic peptides having the minimum number of amino acid residues for antigenicity, through oligopeptides, up to proteins. The peptide may be a

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recombinant peptide expressed from a transformed cell, or could be a synthetic peptide produced by chemical synthesis.

Antibody specific to a peptide of the present invention can be raised using the peptide. The antibody may be used in quality control testing of batches of the peptides; purification of a peptide or viral lysate; epitope mapping; when labelled, as a conjugate in a competitive type assay, for antibody detection; and in antigen detection assays.

Polyclonal antibody against a peptide of the present invention may be obtained by injecting a peptide, optionally coupled to a carrier to promote an immune response, into a mammalian host, such as a mouse, rat, sheep or rabbit, and recovering the antibody thus produced. The peptide is generally administered in the form of an injectable formulation in which the peptide is admixed with a physiologically acceptable diluent. Adjuvants, such as Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA), may be included in the formulation. The formulation is normally injected into the host over a suitable period of time, plasma samples being taken at appropriate intervals for assay for anti-HCV viral antibody. When an appropriate level of activity is obtained, the host is bled. Antibody is then extracted and purified from the blood plasma using standard procedures, for example, by protein A or

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ion-exchange chromatography.

Monoclonal antibody against a peptide of the present invention may be obtained by fusing cells of an immortalising cell line with cells which produce antibody against the viral of topographically related peptide, and culturing the fused immortalised cell line. Typically, a non-human mammalian host, such as a mouse or rat, is inoculated with the peptide. After sufficient time has elapsed for the host to mount an antibody response, antibody producing cells, such as the splenocytes, are removed. Cells of an immortalising cell line, such as a mouse or rat myeloma cell line, are fused with the antibody producing cells and the resulting fusions screened to identify a cell line, such as a hybridoma, that secretes the desired monoclonal antibody. The fused cell line may be cultured and the monoclonal antibody purified from the culture media in a similar manner to the purification of polyclonal antibody.

Diagnostic assays based upon the present invention may be used to determine the presence of absence of HCV infection, and the HCV type involved. They may also be used to monitor treatment of such infection, for example in interferon therapy.

In an assay for the diagnosis of viral infection, there are basically three distinct approaches that can be adopted involving the detection of viral nucleic acid, viral antigen or viral antibody respectively. Viral

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nucleic acid is generally regarded as the best indicator of the presence of the virus itself and would identify materials likely to be infectious. However, the detection of nucleic acid is not usually as straightforward as the detection of antigens or antibodies since the level of target can be very low. Viral antigen is used as a marker for the presence of virus and as an indicator of infectivity. Depending upon the virus, the amount of antigen present in a sample can be very low and difficult to detect. Antibody detection is relatively straightforward because, in effect, the host immune system is amplifying the response to an infection by producing large amounts of circulating antibody. The nature of the antibody response can often be clinically useful, for example IgM rather than IgG class antibodies are indicative of a recent infection, or the response to a particular viral antigen may be associated with clearance of the virus. Thus the exact approach adopted for the diagnosis of a viral infection depends upon the particular circumstances and the information sought. In the case of HCV, a diagnostic assay may embody any one of these three approaches.

In any assay for the diagnosis of HCV involving detection of viral nucleic acid, the method may comprise hybridising viral RNA present in a test sample, or cDNA synthesised from such viral RNA, with a DNA sequence corresponding to the nucleotide sequences of the present

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invention or encoding a peptide of the invention, and screening the resulting nucleic acid hybrids to identify any HCV viral nucleic acid. The application of this method is usually restricted to a test sample of an appropriate tissue, such as a liver biopsy, in which the viral RNA is likely to be present at a high level. The DNA sequence corresponding to a nucleotide sequence of the present invention or encoding a peptide of the invention may take the form of an oligonucleotide or a cDNA sequence optionally contained within a plasmid. Screening of the nucleic acid hybrids is preferably carried out by using a labelled DNA sequence. Preferably the peptide of the present invention is part of an oligonucleotide wherein the label is situated at a sufficient distance from the peptide so that binding of the peptide to the viral nucleic acid is not interfered with by virtue of the label being too close to the binding site. One or more additional rounds of screening of one kind or another may be carried out to characterise further the hybrids and thus identify any HCV viral nucleic acid. The steps of hybridisation and screening are carried out in accordance with procedures known in the art.

A further method for the detection of viral nucleic acid involves amplification of a viral DNA using polymerase chain reaction (PCR). The primers chosen may be specific to the HCV type sequence of interest, so that amplification occurs only with that particular HCV type.



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Also the size and number of amplified copy sequences may be characteristic of particular HCV types, or they may have characteristic restriction patterns with chosen endonucleases.

In an assay for the diagnosis of HCV involving detection of viral antigen or antibody, the method may comprise contacting a test sample with a peptide of the present invention or a polyclonal or monoclonal antibody against the peptide and determining whether there is any antigen-antibody binding contained within the test sample. For this purpose, a test kit may be provided comprising a peptide, as defined herein, or a polyclonal or monoclonal antibody thereto and means for determining whether there is any binding with antibody or antigen respectively contained in the test sample to produce an immune complex. The test sample may be taken from any of appropriate tissue or physiological fluid, such as blood (serum or plasma), saliva, urine, cerebrospinal fluid, sweat, tears or tissue exudate. If a physiological fluid is obtained, it may optionally be concentrated for any viral antigen or antibody present.

A variety of assay formats may be employed. The peptide can be used to capture selectively antibody against HCV from solution, to label selectively the antibody already captured, or both to capture and label the antibody. In addition, the peptide may be used in a variety of homogeneous assay formats in which the antibody

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reactive with the peptide is detected in solution with no separation of phases.

The types of assay in which the peptide is used to capture antibody from solution involve immobilization of the peptide on to a solid surface. This surface should be capable of being washed in some way. Examples of suitable surfaces include polymers of various types (moulded into microtitre wells; beads; dipsticks of various types; aspiration tips; electrodes; and optical devices), particles (for example latex; stabilized red blood cells; bacterial or fungal cells; spores; gold or other metallic or metal-containing sols; and proteinaceous colloids) with the usual size of the particle being from 0.02 to 5 microns, membranes (for example of nitrocellulose; paper; cellulose acetate; and high porosity/high surface area membranes of an organic or inorganic material).

The attachment of the peptide to the surface can be by passive adsorption from a solution of optimum composition which may include surfactants, solvents, salts and/or chaotropes; or by active chemical bonding. Active bonding may be through a variety of reactive or activatable functional groups which may be exposed on the surface (for example condensing agents; active acid esters, halides and anhydrides; amino, hydroxyl, or carboxyl groups; sulphhydryl groups; carbonyl groups; diazo groups; or unsaturated groups). Optionally, the active bonding may be through a protein (itself attached to the surface passively or through active bonding), such

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as albumin or casein, to which the viral peptide may be chemically bonded by any of a variety of methods. The use of a protein in this way may confer advantages because of isoelectric point, charge, hydrophilicity or other physico-chemical property. The viral peptide may also be attached to the surface (usually but not necessarily a membrane) following electrophoretic separation of a reaction mixture, such as immunoprecipitation.

In the present invention it is preferred to provide blocking peptides which block any cross-reactivity and leave only those HCV antibodies in the sample which will react solely with the type of antigen present in that particular test location. For example, a test location intended to detect HCV-6 will be blocked by a blocking mixture comprising HCV-1 to 5 peptides which will react with all antibodies having reactivity to HCV types 1 to 5 and leave antibodies having only type 6 reactivity.

After contacting the surface bearing the peptide with a test sample (in the presence of a blocking mixture if required), allowing time for reaction, and, where necessary, removing the excess of the sample by any of a variety of means, (such as washing, centrifugation, filtration, magnetism or capillary action) the captured antibody is detected by any means which will give a detectable signal. For example, this may be achieved by use of a labelled molecule or particle as described above which will react with the captured antibody (for example protein A or protein G and the like; anti-species or

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anti-immunoglobulin-sub-type; rheumatoid factor; or antibody to the peptide, used in a competitive or blocking fashion), or any molecule containing an epitope contained in the peptide. In the present invention, it is preferred to add an anti-human IgG conjugated to horseradish peroxidase and then to detect the bound enzyme by reaction with a substrate to generate a colour.

The detectable signal may be produced by any means known in the art such as optical or radioactive or physico-chemical and may be provided directly by labelling the molecule or particle with, for example, a dye, radiolabel, fluorescent, luminescent, chemiluminescent, electroactive species, magnetically resonant species or fluorophore, or indirectly by labelling the molecule or particle with an enzyme itself capable of giving rise to a measurable change of any sort. Alternatively the detectable signal may be obtained using, for example, agglutination, or through a diffraction or birefringent effect if the surface is in the form of particles.

Assays in which a peptide itself is used to label an already captured antibody require some form of labelling of the peptide which will allow it to be detected. The labelling may be direct by chemically or passively attaching for example a radiolabel, magnetic resonant species, particle or enzyme label to the peptide; or indirect by attaching any form of label to a molecule which will itself react with the peptide. The chemistry of bonding a label to the peptide can be directly through

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a moiety already present in the peptide, such as an amino group, or through an intermediate moiety, such as a maleimide group. Capture of the antibody may be on any of the surfaces already mentioned in any reagent including passive or activated adsorption which will result in specific antibody or immune complexes being bound. In particular, capture of the antibody could be by anti-species or anti-immunoglobulin-sub-type, by rheumatoid factor, proteins A, G and the like, or by any molecule containing an epitope contained in the peptide.

The labelled peptide may be used in a competitive binding fashion in which its binding to any specific molecule on any of the surfaces exemplified above is blocked by antigen in the sample. Alternatively, it may be used in a non-competitive fashion in which antigen in the sample is bound specifically or non-specifically to any of the surfaces above and is also bound to a specific bi- or poly-valent molecule (e.g. an antibody) with the remaining valencies being used to capture the labelled peptide.

Often in homogeneous assays the peptide and an antibody are separately labelled so that, when the antibody reacts with the recombinant peptide in free solution, the two labels interact to allow, for example, non-radiative transfer of energy captured by one label to the other label with appropriate detection of the excited second label or quenched first label (e.g. by fluorimetry, magnetic resonance or enzyme measurement). Addition of

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either viral peptide or antibody in a sample results in restriction of the interaction of the labelled pair and thus in a different level of signal in the detector.

A further possible assay format for detecting HCV antibody is the direct sandwich enzyme immunoassay (EIA) format. An antigenic peptide is coated onto microtitre wells. A test sample and a peptide to which an enzyme is coupled are added simultaneously. Any HCV antibody present in the test sample binds both to the peptide coating the well and to the enzyme-coupled peptide. Typically, the same peptide are used on both sides of the sandwich. After washing, bound enzyme is detected using a specific substrate involving a colour change.

It is also possible to use IgG/IgM antibody capture ELISA wherein an antihuman IgG and/or IgM antibody is coated onto a solid substrate. When a test sample is added, IgG and/or IgM present in the sample will then bind to the antihuman antibody. The bound IgG and/or IgM represents the total population of those antibodies. A peptide of the present invention will bind only to those IgG and/or IgM antibodies that were produced in response to the antigenic determinant(s) present in the peptide i.e. to those antibodies produced as a result of infection with the type of HCV from which the peptide was derived. For detection of the peptide/antibody complex the peptide may itself have been labelled directly or, after interaction with the captured antibodies, the peptide may

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be reacted with a labelled molecule that binds to the peptide.

It can thus be seen that the peptides of the present invention may be used for the detection of HCV infection in many formats, namely as free peptides, in assays including classic ELISA, competition ELISA, membrane bound EIA and immunoprecipitation. Peptide conjugates may be used in amplified assays and IgG/IgM antibody capture ELISA.

An assay of the present invention may be used, for example, for screening donated blood or for clinical purposes, for example, in the detection, typing and monitoring of HCV infections. For screening purposes, the preferred assay formats are those that can be automated, in particular, the microtitre plate format and the bead format. For clinical purposes, in addition to such formats, those suitable for smaller-scale or for single use, for example, latex assays, may also be used. For confirmatory assays in screening procedures, antigens may be presented on a strip suitable for use in Western or other immunoblotting tests.

As indicated above, assays used currently to detect the presence of anti-HCV antibodies in test samples, particularly in screening donated blood, utilise antigenic peptides obtained from HIV type 1 only and such antigens do not reliably detect other HCV genotypes. Accordingly, it is clearly desirable to supplement testing for HIV-1 with testing for all other genotypes, for example, types

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2, 3, 4, 5 and 6 and also any further genotypes that may be discovered.

In particular, the invention allows blood donor screening by conventional assays (using HCV type 1 encoded antigens) to be supplemented with a second test that contains oligopeptides corresponding to antigenic regions found for example in the NS5 sequence of HCV-4 or HCV-6 , or the NS4 sequence of HCV-4, HCV-5 or HCV-6.

To test for a spectrum of genotypes, there may be provided a series of assay means each comprising one or more antigenic peptides from one genotype of HCV, for example, a series of wells in a microtitre plate, or an equivalent series using the bead format. Such an assay format may be used to determine the type of HCV present in a sample. Alternatively, or in addition, an assay means may comprise antigenic peptides from more than one type, for example, a microwell or bead may be coated with peptides from more than one type.

Oligopeptides corresponding to the antigenic regions of HCV-4, HCV-5 or HCV-6 may also be used separately to distinguish individuals infected with these different HCV types. Such an assay could be in the format of an indirect enzyme immunoassay (EIA) that used sets of wells or beads coated with oligopeptides of the antigenic regions for HCV types 4, 5 and 6. Minor degrees of cross-reactivity, should they exist, can be absorbed out by dilution of the test serum in a diluent that contained blocking amounts of soluble heterologous-type



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oligopeptides, to ensure that only antibody with type-specific antibody reactivity bound to the solid phase.

It may be advantageous to use more than one HCV antigen for testing, in particular, a combination comprising at least one antigenic peptide derived from the structural region of the genome and at least one antigenic peptide derived from the non-structural region, especially a combination of a core antigen and at least one antigen selected from the NS3, NS4 and NS5 regions. The wells or beads may be coated with the antigens individually. It may be advantageous, however, to fuse two or more antigenic peptides as a single polypeptide, preferably as a recombinant fusion polypeptide. Advantages of such an approach are that the individual antigens can be combined in a fixed, predetermined ratio (usually equimolar) and that only a single polypeptide needs to be produced, purified and characterised. One or more such fusion polypeptides may be used in an assay, if desired in addition to one or more unfused peptides. It will be appreciated that there are many possible combinations of antigens in a fusion polypeptide, for example, a fusion polypeptide may comprise a desired range of antigens from one type only, or may comprise antigens from more than one type.

To obtain a polypeptide comprising multiple peptide antigens by an expression technique, one approach is to fuse the individual coding sequences into a single open reading frame. The fusion should, of course, be carried

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out in such a manner that the antigenic activity of each component peptide is not significantly compromised by its position relative to another peptide. Particular regard should of course be had for the nature of the sequences at the actual junction between the peptides. The resulting coding sequence can be expressed, for example, as described above in relation to recombinant peptides in general. The methods by which such a fusion polypeptide can be obtained are known in the art, and the production of a recombinant fusion polypeptide comprising multiple antigens of a strain of HCV type 1 is described in GB-A-2 239 245. Peptide conjugates may be used in amplified assays and IgG/IgM antibody capture ELISA.

The peptide of the present invention may be incorporated into a vaccine formulation for inducing immunity to HCV in man. The vaccine may include antigens of HCV types 1 to 6. For this purpose the peptide may be presented in association with a pharmaceutically acceptable carrier.

For use in a vaccine formulation, the peptide may optionally be presented as part of an hepatitis B core fusion particle, as described in Clarke et al (Nature, 1987, 330, 381-384), or a polylysine based polymer, as described in Tam (PNAS, 1988, 85, 5409-5413).

Alternatively, the peptide may optionally be attached to a particulate structure, such as liposomes or ISCOMS.

Pharmaceutically acceptable carriers for the vaccine include liquid media suitable for use as vehicles to

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introduce the peptide into a patient. An example of such liquid media is saline solution. The peptide may be dissolved or suspended as a solid in the carrier.

The vaccine formulation may also contain an adjuvant for stimulating the immune response and thereby enhancing the effect of the vaccine. Examples of adjuvants include aluminium hydroxide and aluminium phosphate.

The vaccine formulation may contain a final concentration of peptide in the range from 0.01 to 5 mg/ml, preferably from 0.03 to 2 mg/ml. The vaccine formulation may be incorporated into a sterile container, which is then sealed and stored at a low temperature, for example 4°C, or may be freeze-dried.

In order to induce immunity in man to HCV, one or more doses of the vaccine formulation may be administered. Each dose may be 0.1 to 2 ml, preferably 0.2 to 1 ml. A method for inducing immunity to HCV in man, comprises the administration of an effective amount of a vaccine formulation, as hereinbefore defined.

The present invention also provides the use of a peptide as herein defined in the preparation of a vaccine for use in the induction of immunity to HCV in man.

Vaccines of the present invention may be administered by any convenient method for the administration of vaccines including oral and parenteral (e.g. intravenous, subcutaneous or intramuscular) injection. The treatment may consist of a single dose of vaccine or a plurality of doses over a period of time.

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Description of the Drawings

Examples of the invention will now be described by way of example only.

Figure 1 is a comparison of inferred amino acid sequences of part of the NS5 region of HCV types 4 and 6 (in comparison to type 1a) from Example 1. The number of sequences compared are shown in the second column. Single amino acid codes are used. The position and frequency of variability within an HCV type is indicated by a subscript;

Figure 2 gives corresponding DNA nucleotide sequences in the NS5 region of HCV-4 (three sequences) and of HCV-6 (one sequence);

Figure 3 is a phylogenetic analysis of NS5 sequences from 67 isolates of HCV, showing major HCV types (numbered 1-6) and subtypes (designated a,b,c) and demonstrating that HCV-4 and HCV-6 are distinct types. The sequence distance is proportional to the spacing on the tree according to the indicated scale.

Figure 4 shows DNA primer sequences as used in Example 2 for the PCR amplification of two regions of the NS4 region of HCV-4, HCV-5 and HCV-6; and

Figure 5 shows DNA and amino acid sequences for two partial regions of the NS4 region of HCV-4, HCV-5 and HCV-6 deduced from the nucleotide sequences elucidated in Example 2; for comparison the corresponding regions of HCV-1, 2 and 3 are given, HCV-3 regions 1 and 2 corresponding to amino acids 1691-1708 and 1710-1728

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respectively of Figure 9b of WO93/10239 (see also Simmonds et al., 1993, J. Clin. Microb. 31:1493).

EXAMPLE 1 (HCV-4 and HCV-6; NS5 region sequences)

**Samples.** Plasma from a total of 16 HCV-infected blood donors in Scotland, Egypt and Hong Kong and from a patient with chronic hepatitis in Lebanon were used for analysis of NS5 sequences of types 4 and 6.

**Nucleotide sequence analysis.** To obtain sequences in the NS5 region, viral RNA was reverse transcribed and amplified by polymerase chain reaction (PCR) in a single reaction with previously published primers thought to be highly conserved amongst different variants of HCV (Enomoto et al. 1990). For some sequences, a second PCR was carried out with primers 554 and 555 (Chan et al., 1992b) in combination with two new primers, 122 (sense orientation; 5' CTC AAC CGT CAC TGA GAG AGA CAT 3') and 123 (anti-sense; 5' GCT CTC AGG TTC CGC TCG TCC TCC 3'). Product DNA was phosphorylated, purified and cloned into the SmaI site of pUC19 (Yanisch-Perron et al., 1985) following the procedures described in Simmonds & Chan, 1993. Alternatively, amplified DNA was purified and directly sequenced as described in Simmonds et al., 1990 and Cha et al., 1992. These methods allowed comparison of a 222bp fragment of DNA homologous to positions 7975 to 8196 in the prototype virus (numbered as in Choo et al., 1991). The results are shown in Figures 1 and 2.

**Nucleotide sequence comparisons.** Nucleotide

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sequences were aligned using the CLUSTAL V program (Higgins et al. 1992) as implemented in the GDE sequence analysis package. Distances between pairs of sequences were estimated using the DNADIST program of the PHYLIP package (version 3,4) kindly provided by Dr. J. Felsenstein (Felsenstein, 1991), using a model which allow different rates of transition and tranversion and different frequencies of the four nucleotides (Felsenstein, 1991). Phylogenetic trees were constructed using the neighbour-joining algorithm on the previous sets of pairwise distances (Saitou et al. 1987) using the PHYLIP program, NEIGHBOR. The phylogenetic tree shown in Figure 3 is unrooted. Equivalent phylogenetic relationships were also found in a maximum likelihood analysis (PHYLIP program DNAMI; data not shown), and 200 bootstrap replicates of neighbour-joining trees (PHYLIP programs SEQBOOT and CONSENSE).

EXAMPLE 2 (HCV-4, -5 and -6; NS4 region sequences)

Attempts have been made to isolate DNA sequences from the NS4 region of Hepatitis C virus (HCV) types 4, 5 and 6 using PCR amplification. The decision was made to use primers which contained restriction sites, thereby allowing the cloning of the PCR products via cohesive end cloning. New primers were also designed from relatively conserved regions of the HCV NS4 gene. The cloning strategy involved several particular steps, as follows:

(i) Klenow repair. The termini of the amplified DNA

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were repaired with Klenow DNA polymerase to ensure that the ends which contained the restriction sites were complete.

- (ii) Kinasing of termini. The PCR product termini were phosphorylated using T4 polynucleotide kinase. This allowed self-ligation of the products in a concatemerization step.
- (iii) Concatemerization of the PCR products. The DNA fragments were ligated together to form long concatemeric arrays. This step internalized the restriction sites encoded in the ends of the primers which greatly increased the efficiency of the cleavage step.
- (iv) Restriction digestion. The PCR products were digested overnight with the required restriction enzyme to expose the cohesive ends.
- (v) Ligation to the plasmid vector.

General procedures and reagents are described in Maniatis et al. "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor: New York.

a) PCR Amplification of NS4 sequences

After cDNA synthesis using primer 007, three rounds of PCR amplification were carried out. Types 4 and 5 were amplified by one round using 007 and 435, followed by two rounds using 5351 and 5943 (which both encode BamHI

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restriction sites). Type 6 sequences were amplified by one round using 007 plus 253, 281 and 221, followed by two rounds using 5351 and 5943. The products of a minimum of 5 third round reactions were pooled for the cloning steps. The efficiency of the final ligation into the vector appeared to be dependant on a high concentration of the amplified DNA. The primer sequences are listed in Figure 4.

b) Cloning

The PCR products were isolated by excision from a conventional agarose gel (0.5x tris acetate EDTA (TAE)). The DNA was reclaimed from the agarose by centrifugation through glass wool. The eluates were pooled in order to increase the total amount of DNA. The DNA was retrieved from the TAE eluate by ethanol precipitation. (The pelleted DNA contained some chemically inert debris from the agarose but this did not interfere with the following steps prior to ligation into the vector. Magic prep columns (Promega) could be used, but ethanol precipitation is simpler, cheaper and more efficient if dealing with a large volume of TAE eluate.)

c) Klenow repair

The precipitated DNA pellets were resuspended in a 50ul Klenow reaction mixture comprising:.  
5ul polynucleotide kinase buffer (10x),



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0.5ul 3.3mM dNTPs (final concentration 33uM),  
at least 100ng of purified PCR fragment,  
distilled water to 50ul, and  
5 units Klenow DNA polymerase.

(10x means that the reagent was added at 10 times the  
desired final concentration in the reaction mixture)  
Incubation was carried out for 30 min at 37°C, followed by  
heat inactivation for 10 min at 75°C.

The Klenow reaction repairs the ends where the BamHI  
restriction sites are located. T4 DNA polymerase should  
not be used for this reaction as it will remove the sites  
by its exonuclease activity.

d) Kinasing of ends

To the above reaction mixture, the following were  
added:

5ul 100mM rATP, and  
10 units T4 polynucleotide kinase.

This reaction mixture was incubated at 37°C for 30-60 min  
and heat inactivated as before.

e) Concatemerization

The PCR products were then concatemerized to  
internalise the BamHI restriction sites within large  
multimers.

To the phosphorylation reaction, the following were added:  
6ul 10x ligase buffer, and

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5 units of T4 DNA ligase.

The ligation was incubated overnight at 15°C. The concatemerization reaction was heat inactivated as described previously.

f) Restriction digestion

The concatemerized PCR products were digested into monomers using BamHI restriction enzyme which simultaneously exposed the cohesive termini. The following were added to the heat inactivated ligation reaction mixture:

6ul 10x B buffer (Boehringer), and  
10-20 units BamHI.

The digestion mixture was incubated overnight at 37°C. Although the enzyme is not totally inactivated by heat, the reaction mixture was heat treated as before anyway.

At this point, the DNA was purified by a Magic prep column to remove cleaved ends. The DNA was eluted from the Magic prep column in 10ul in order to be as concentrated as possible.

g) Ligation to vector

100ng of bacterial plasmid vector pUC18 was used in the ligation. The plasmid vector DNA had been BamHI-cleaved and purified using "Geneclean" (Bio 101), but not dephosphorylated. (We found that the

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dephosphorylation reaction lowered the ligation efficiency of the vector to a great extent.) It was planned that blue-white colour selection as described hereafter would be sufficient to identify clones. The ligation reaction mixture contained the following:

10ul of purified insert DNA produced as above  
5ul plasmid vector DNA,  
1.5ul 10x ligase buffer, and  
1 unit of ligase.

The reaction mixture was incubated overnight at 15°C.

h) Transformation of E. coli

Bacterial transformations were carried out using the cell strain, XL-1 Blue (Stratagene). Cells were made competent for transformation by standard calcium chloride methods and stored quick-frozen in a glycerol/calcium chloride suspension in 200ul aliquots. 3ul of the ligation reaction product were used to transform 100ul of rapidly thawed competent XL-1 Blue cells (10 min on ice, 2 min at 42°C, 1 hour at 37°C with shaking after addition of 1 ml of L broth). The cells were plated in 200ul aliquots onto L agar plates containing X-Gal (20ug/ml), IPTG (0.1mM), Ap (50ug/ml) and Tet (12.5 ug/ml). The presence of the chemicals IPTG (isopropyl-β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the media produces a blue colour in colonies which contain

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plasmids such as the pUC series which encode the lacZ enzymic peptide. The insertion of cloned DNA into the polylinker region of these plasmids interrupts the lacZ peptide sequence, thereby destroying the ability of the plasmid to produce the enzyme. Bacterial colonies which contain recombinant plasmids will, therefore, be white.

i) Analysis of white colonies

Blue-white selection identified cell colonies which contained recombinant plasmids. These colonies were picked and DNA was prepared from them by mini-plasmid preparations. Digestion of the DNA with BamHI confirmed that the plasmids contained cloned inserts. The plasmid DNA was purified by glassmilk and sequenced using the USB Sequenase kit with M13 forward and reverse primers.

j) Discussion

Although this protocol contains a large number of steps, it is simple to execute. Further work has found that the method has a high degree of reproducibility. Theoretically, this cloning strategy should not work if the PCR product contains an internal BamHI site. However, the NS4 sequence which was obtained for type 6 did contain such an internal site; and there are no obvious reasons why foreshortened type 6 sequences were not in fact the predominant product of the cloning experiment.

Figure 5 shows the DNA and amino acid sequences for two regions of NS4 in respect of HCV types 1 to 3 (for comparison) and types 4 to 6.

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EXAMPLE 3 (Synthesis of NS4 peptides)

The following peptides within the NS4 region of HCV types 1 to 6 were synthesised. Types 4 to 6 are novel sequences according to the present invention, whereas types 1 to 3 are presented for comparison purposes and use in a complete HCV serotyping assay.

HCV type 1	[H <sub>2</sub> N-KPAIIPDREVLVREFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL031)
HCV type 1	[H <sub>2</sub> N-KPAVIPDREVLVREFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL033)
HCV type 1	[H <sub>2</sub> N-RPAVIPDREVLVQEFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL036)
HCV type 1	[H <sub>2</sub> N-RPAVVPDREVLVQEFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL035)
HCV type 1	[H <sub>2</sub> N-ECSQHLPYIEQGMLAEQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL037Q)
HCV type 1	[H <sub>2</sub> N-ECSQHLPYIEQGMALAEQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL038Q)
HCV type 2	[H <sub>2</sub> N-RVVVTPDKEILYEAFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL039)
HCV type 2	[H <sub>2</sub> N-ECASRAALIEEGQRIAEML] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL041)
HCV type 2	[H <sub>2</sub> N-ECASKAALIEEGQMAEML] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL040)
HCV type 3	[H <sub>2</sub> N-KPALVPDKEVLVQQYDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL042)
HCV type 3	[H <sub>2</sub> N-ECSQAAPYIEQAQVIAHQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL044)
HCV type 4	[H <sub>2</sub> N-QPAVIPDREVLVQQFEDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL034)
HCV type 4	[H <sub>2</sub> N-ECSKHLPLVEHGLQLAEQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL028)
HCV type 5	[H <sub>2</sub> N-RPAIIPDREVLVQQFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL024)
HCV type 5	[H <sub>2</sub> N-ECSTSLPYMDEARAIAGQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL029)
HCV type 6	[H <sub>2</sub> N-KPAVVPDREILVQQFDEM] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL025)
HCV type 6	[H <sub>2</sub> N-ECSRHIPYLAEGQQIAEQF] <sub>8</sub> K <sub>4</sub> K <sub>2</sub> K-COOH	(MDL022)

Below is a typical example of the synthesis of one of the peptides.

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(a) Synthesis of multiple antigenic peptide MDL029.

In order to work successfully, the serotyping assay requires that peptides are synthesized on a special resin support, bearing the multiple antigen peptide core (K<sub>4</sub>K<sub>2</sub>K) as developed by Tam (Tam J.P., 1988, Proc.Natl. Acad. Sci.USA., 85:5409:5413). All peptides were synthesized on an Applied Biosystems model 432A Synergy peptide synthesizer running FASTmoc™ chemistry. Peptide synthesis was achieved using the standard run program without modification. All reagents used were supplied by Applied Biosystems Limited (Kelvin Close, Birchwood Science Park, Warrington, UK). The MAP resin was hepta-lysyl (K<sub>4</sub>K<sub>2</sub>K) core on a polyoxyethylene/polystyrene co-polymer with an HMP linker and β-alanine internal reference amino acid. The N-α-amino group of all amino acids was protected by the 9-fluorenylmethoxycarbonyl (Fmoc) group. Amino acids with reactive side groups were protected as follows;

<u>Amino Acid</u>	<u>Code</u>	<u>Protecting Group</u>
glutamine	Q	tert. Butyl ester (OtBu)
cysteine	C	trityl (Trt)
serine	S	tert. Butyl (tBu)
threonine	T	tert. Butyl (tBu)
tyrosine	Y	tert. Butyl (tBu)
aspartic acid	D	tert. Butyl ester (OtBu)
arginine	R	2,2,5,7,8-Pentamethylchroman -6-sulphonyl (Pmc)

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The progress of the synthesis was monitored by measuring the conductivity of the coupling and deprotection mixtures. There are no abnormalities in the conductivity trace and the synthesis was considered successful.

Following the synthesis, the fully protected peptide resin was transferred to a conical 50mL capacity polypropylene tube and treated with thioanisole (0.15mL), ethandithiol (0.15mL) and trifluoroacetic acid (TFA; 2.7mL). The mixture was stirred for three hours at room temperature, then filtered through glass wool in a Pasteur pipette, the filtrate dropping into 20mL tert. butylmethylether (TMBE) contained in a glass screw-capped bottle, whereupon the peptide precipitated. The tube was centrifuged and the liquor aspirated, leaving the peptide pellet in the tube. Peptide was washed with three further 20mL aliquots of TMBE, using a spatula to dis-aggregate the pellet and centrifugation to recover the peptide. The peptide was finally dried at room temperature in vacuo.

To analyse the purity of the peptide, a small sample was dissolved in purified water and submitted to analysis by reverse phase high-performance liquid chromatography (HPLC). The reverse phase column was 250 x 4.6 mm containing a C<sub>4</sub> matrix. Peptide was eluted with a gradient of 3.5% acetonitrile to 70% acetonitrile over 20 minutes at a flow rate of 1.5mL min<sup>-1</sup>. The eluant was monitored at 214nm to detect peptide.

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EXAMPLE 4(Assay for the determination of HCV serotypes 1-6)

We have developed an assay based on selective competition, which is capable of distinguishing the serotype of HCV to which antibodies present in a biological sample have been produced. Typically the sample will be serum or plasma from a human with confirmed hepatitis C infection.

The assay relies on the selectivity of 17 different synthetic peptides covering variable sequences within the NS4 protein of hepatitis C virus types 1,2,3,4,5 & 6. Whilst there is a degree of cross-reactivity between antisera raised to NS4 of an HCV serotype and the homologous region of other serotypes, this can be blocked without completely removing the true reactivity. In view of this, we have developed an assay format involving coating wells of a microtitre plate with an equal amount of all 17 peptides. Samples are tested in eight duplicate wells which each receive a different mixture of blocking peptides. Only one testing well and the un-completed control well should contain colouration at the end of the assay protocol, thereby identifying the serotype of the infecting hepatitis C virus.

Plates for serotyping assays are prepared by coating approximately equimolar amounts of each of the peptides onto polystyrene microwell plates. Peptides are dissolved



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in purified water and a mixture is made at the following concentration:

MDL031 )	
MDL033 )	
MDL036 )	25 ng/ml
MDL035 )	
MDL037Q)	
MDL038Q)	

MDL039 )	
MDL041 )	
MDL040 )	
MDL042 )	
MDL044 )	
MDL034)	50 ng/ml
MDL028 )	
MDL024 )	
MDL029 )	
MDL025 )	
MDL022 )	

Although strictly speaking each microwell need only contain peptide of the type which it is intended to detect, it is more convenient to coat each microwell with all six antigen types.

Peptides were allowed to bind to the plates by adding 100 ul of the peptide mixture into each well of the plate and incubating at +4°C overnight.

To provide sufficient differentiation between the various serotypes, it is then necessary to add competing heterologous peptides to the sample being tested. Competing solutions are made up in assay sample diluent to give a 100-fold excess of competing peptide, relative to the coating concentration. The different competing solutions are classified according to the serotype for

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which they are blocking, i.e. Competing Solution 1 contains peptides for types 2-5. Competing solutions are made to have the excess required in 10ul. The competing solutions are as follows:

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<u>Competing solution</u>	<u>Peptide</u>	<u>Concentration</u>
1	MDL039	50ug/ml
	MDL041	"
	MDL040	"
	MDL042	"
	MDL044	"
	MDL034	"
	MDL028	"
	MDL024	"
	MDL029	"
	MDL025	"
	MDL022	"
2	MDL031	25ug/ml
	MDL033	"
	MDL036	"
	MDL035	"
	MDL037Q	"
	MDL038Q	"
	MDL042	50ug/ml
	MDL044	"
	MDL034	"
	MDL028	"
	MDL024	"
	MDL029	"
	MDL025	"
	MDL022	"
3	MDL031	25ug/ml
	MDL033	"
	MDL036	"
	MDL035	"
	MDL037Q	"
	MDL038Q	"
	MDL039	50ug/ml
	MDL040	"
	MDL041	"
	MDL034	"
	MDL028	"
	MDL024	"
	MDL029	"
	MDL025	"
	MDL022	"

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<u>Competing Solution</u>	<u>Peptide</u>	<u>Concentration</u>
4	MDL031	25ug/ml
	MDL033	"
	MDL036	"
	MDL035	"
	MDL037Q	"
	MDL038Q	"
	MDL039	50ug/ml
	MDL040	"
	MDL041	"
	MDL042	"
	MDL044	"
	MDL024	"
	MDL029	"
	MDL025	"
	MDL022	"
5	MDL031	25ug/ml
	MDL033	"
	MDL036	"
	MDL035	"
	MDL037Q	"
	MDL038Q	"
	MDL039	50ug/ml
	MDL040	"
	MDL041	"
	MDL034	"
	MDL028	"
	MDL042	"
	MDL044	"
	MDL025	"
	MDL022	"
6	MDL031	25ug/ml
	MDL033	"
	MDL036	"
	MDL035	"
	MDL037Q	"
	MDL038Q	"
	MDL039	50ug/ml
	MDL040	"
	MDL041	"
	MDL034	"
	MDL028	"
	MDL024	"
	MDL029	"
	MDL042	"
	MDL044	"

The protocol for using the serotyping assay is as follows.

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- 1) Add 180ul sample of diluent to each well.
- 2) Add 10ul blocking peptides to relevant wells.
- 3) Add 10ul sample to each of six wells.
- 4) Mix plate and incubate at 37°C for 1 hour.
- 5) Wash wells three times.
- 6) Add 100 ul conjugate to each well.
- 7) Incubate at 37°C for 1 hour.
- 8) Wash wells three times.
- 9) Add 100ul TMB solution (including  
3,3',5,5'-tetramethylbenzidine, hydrogen peroxide,  
buffers etc).
- 10) Incubate at 37°C for 30 minutes.
- 11) Stop reaction with sulphuric acid; and
- 12) Read optical density at 450nm/690nm.

Samples known to contain anti-HCV antibodies are tested at a dilution of 1/20 with 100 fold excess of competing peptides, in a total volume of 200ul as described above. Following incubation, sample is removed and the wells are washed. An anti-human immunoglobulin G conjugated to horseradish peroxidase is added, and binds to any captured anti-HCV antibodies. Bound antibody is then visualised by removing the enzyme conjugate and adding a substrate and chromogen, which, in the presence of enzyme, converts from a colourless to a coloured solution. The intensity of the colour can be measured and is directly proportional to the amount of enzyme present. Results on certain samples are given in Table 1.

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EXAMPLE 5(Assay for the determination of HCV serotypes 4-6)

An alternative to the assay format in Example 4 has been developed. This assay is more limited in scope using only the peptides for types 4,5 & 6. For some samples, the more comprehensive assay may give erroneous results, due to greater cross-reactivity with type 1 peptides. The protocol for performing the more restricted assay is identical to that given in Example 4.

Plates for the HCV types 4-6 assay are prepared as described in Example 4 with the only difference being the reduced number of peptides used. Peptides are dissolved in purified water and a mixture is made at the following concentration:

MDL034 )	
MDL028 )	
MDL024 )	50 ng/ml
MDL029 )	
MDL025 )	
MDL022 )	

Peptides are allowed to bind to the plates by adding 100 ul of the peptide mixture into each well of the plate and incubating at +4°C overnight.

To provide sufficient differentiation between the various serotypes, it is then necessary to add competing heterologous peptides with the sample being tested. Competing solutions are made up in assay sample diluent to give a 100-fold excess of competing peptide, relative to the coating concentration.

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The different competing solutions are classified according to the serotype for which they are blocking, i.e.

Competing Solution 4 contains peptides for types 5 & 6.

Competing solutions are made to have the excess required in 10ul. The competing solutions are as follows:

<u>Competing Solution</u>	<u>Peptide</u>	<u>Concentration</u>
4	MDL024	50 ug/ml
	MDL029	"
	MDL025	"
	MDL022	"
5	MDL034	50 ug/ml
	MDL028	"
	MDL025	"
	MDL022	"
6	MDL034	50 ug/ml
	MDL028	"
	MDL024	"
	MDL029	"

Samples known to contain anti-HCV antibodies are tested at a dilution of 1/20 with 100 fold excess of competing peptides, in a total volume of 200ul, as described in Example 4. Following incubation, sample is removed and the wells are washed. An anti-human immunoglobulin G conjugated to horseradish peroxidase is added, and binds to any captured anti-HCV antibodies. Bound antibody is then visualised by removing the enzyme conjugate and adding a substrate and chromogen, which, in the presence of enzyme, converts from a colourless to a coloured solution. The intensity of the colour can be measured and is directly proportional to the amount of enzyme present.

Results on certain samples are given in Table 2.

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TABLE 1

SAMPLE	NO BLOCK	ALL BLOCK	TYPE 1	TYPE 2	TYPE 3	TYPE 4	TYPE 5	TYPE 6	RESULT
Control 1	0.515	0.049	<b>0.530</b>	0.033	0.038	0.038	0.031	0.029	1
Control 2	<b>1.821</b>	0.010	0.028	<b>1.916</b>	0.004	-0.007	0.040	0.005	2
Control 3	<b>2.035</b>	0.066	0.048	0.046	<b>1.933</b>	0.048	0.067	0.044	3
Egypt 3	<b>OVER</b>	0.372	0.346	0.478	0.322	<b>OVER</b>	0.256	0.430	4
C1016116	<b>OVER</b>	0.456	0.635	0.480	0.533	0.533	<b>OVER</b>	0.617	5
Control 6	<b>0.082</b>	0.004	0.007	-0.003	-0.004	-0.012	-0.011	<b>0.067</b>	6
PD372	<b>OVER</b>	0.076	<b>1.497</b>	0.081	0.072	0.040	0.057	0.075	1
PD513	<b>OVER</b>	0.548	0.467	<b>OVER</b>	0.553	0.470	0.525	0.582	2
AD558	<b>OVER</b>	0.031	0.064	0.044	<b>OVER</b>	0.035	0.054	0.048	3
Egypt 37	<b>OVER</b>	0.034	0.064	0.039	0.045	<b>1.473</b>	0.047	0.037	4
C1015921	<b>0.173</b>	0.010	0.017	0.008	0.011	0.005	<b>0.182</b>	0.012	5

Positive results are shown in bold text.



TABLE 2

<u>SAMPLE</u>	<u>NO BLOCK</u>	<u>ALL BLOCK</u>	<u>TYPE 4</u>	<u>TYPE 5</u>	<u>TYPE 6</u>
EGYPT 1	<b>OVER</b>	0.109	<b>1.669</b>	0.184	0.048
EGYPT 2	<b>0.525</b>	0.010	<b>0.514</b>	0.001	-0.006
EGYPT 3	<b>OVER</b>	0.269	<b>OVER</b>	0.329	0.201
EGYPT 4	<b>0.266</b>	-0.001	<b>0.292</b>	-0.006	-0.002
EGYPT 5	<b>1.677</b>	0.090	<b>1.274</b>	0.094	0.086
EGYPT 6	<b>OVER</b>	0.208	<b>0.317</b>	0.041	0.022
EGYPT 7	<b>OVER</b>	0.113	<b>OVER</b>	0.143	0.204
EGYPT 8	<b>OVER</b>	0.085	<b>0.891</b>	0.105	0.098
EGYPT 9	<b>0.051</b>	0.009	<b>0.055</b>	0.014	0.022
EGYPT 10	<b>OVER</b>	0.021	<b>2.023</b>	0.043	0.073
EGYPT 11	<b>0.077</b>	0.024	<b>0.095</b>	0.032	0.048
C1016116	<b>OVER</b>	0.647	0.778	<b>OVER</b>	0.513
C1015921	<b>0.196</b>	0.014	0.009	<b>0.222</b>	0.009
C1132558	<b>0.015</b>	0.007	0.008	<b>0.015</b>	0.007
K904836	<b>OVER</b>	0.073	0.101	<b>OVER</b>	0.071
Control o	<b>0.433</b>	0.029	0.013	0.015	<b>0.314</b>
HK T3950	<b>0.428</b>	0.034	0.017	0.027	<b>0.366</b>
HK T3943	<b>OVER</b>	0.348	0.424	0.342	<b>1.105</b>

Positive results are shown in bold text.

NB. - The preponderance of Type 4 results reflects the relative incidence and availability of samples for types 4, 5, & 6.

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CLAIMS

1. A polynucleotide having a nucleotide sequence unique to hepatitis virus type 4,5 or 6; but excluding 5'NCR and core sequences of type 4.
2. A polynucleotide according to claim 1 wherein the sequence codes for a peptide.
3. A polynucleotide according to claim 2 wherein the peptide is an antigenic peptide.
4. A polynucleotide according to any preceding claim which codes for the NS4 region or a part thereof.
5. A polynucleotide according to claim 4 which codes for the NS4 region of HCV-4 or a part thereof.
6. A polynucleotide according to claim 4 which codes for the NS4 region of HCV-5 or a part thereof.
7. A polynucleotide according to claim 4 which codes for the NS4 region of HCV-6 or a part thereof.
8. A polynucleotide according to any of claims 1 to 3 which codes for the NS5 region or a part thereof.

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9. A polynucleotide according to claim 8 which codes for the NS5 region of HCV-4 or a part thereof.

10. A polynucleotide according to claim 8 which codes for the NS5 region of HCV-6 or a part thereof.

11. A polynucleotide according to claim 1 having a sequence selected from the following:

CAGCCTGCTGTTATCCCTGACCGCGAGGTGCTCTACCAGCAGTTCGACGAAATG  
AGACCTGCCATCATTCCCGATAGAGAGGTGTTGTACCAGCAATTTGATAAGATG  
AAGCCTGCTGTTGTCCCTGATCGCGAGATCTTATACCAGCAGTTTGACGAGATG  
GAGTGTTCCAAACACCTTCCACTAGTCGAGCACGGGTTGCAACTTGCTGAGCAATTC  
GAGTGCTCGACCTCGCTCCCCTATATGGACGAGGCACGTGCTATTGCCGGGCAATTC  
GAGTGCTCTAGGCACATCCCCTACCTCGCTGAGGGCCAGCAGATCGCCGAACAGTTC  
GTCTATCAGTGTTGTAACTGGAGCCCCGAAGCTCGCAAGGCTATTACTGCCCTCACAGAA  
AGACTCTACGTGGGCGGCCCCATGCACAACAGCAAGGGAGACCTTTGTGGGTATCGGAGA  
TGTCGGGCAAGCGGAGTCTTTACGACCAGCTTCGGAAACACGCTGACGTGCTACCTAAAA  
GCCACGGCCGCTATTAGAGCGGCGGGGCTGAGAGACTGCACT  
GTCTATCAGTGTTGTAGTCTGGAGCTCGAGGCTCGCAAGGTTATTACTGCCCTCACGGAA  
AGACTCTACGTGGGCGGCCCCATGCACAATAGCAAGGGAGACCTTTGTGGGTACCGGAGA  
TGCCGGGCAAGCGGAGTCTATACGACCAGCTTCGGAAACACGCTGACGTGCTACCTCAAA  
GCCACAGCCGCTATTAGGGCGGCGGGACTAAAAGACAGCACT  
ATCTATCAGTCTTGCCAGCTGGATCCCGTAGCAAGGAGGGCAGTATCATCCCTGACAGAA  
CGGCTCTACGTAGGCGGCCCCATGGTGAACCTCAAGGGACAGTCATGTGGCTACCGTAGA  
TGCCGCGCCAGTGGGGTGCTGCCCACGAGCATGGGAAACACCATCACGTGCTATCTGAAG  
GCACACGCC---TGCAGGGCGGCCAACATCAAGGACTGTGAC

12. A polynucleotide according to any preceding claim which is a cDNA sequence.

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13. A peptide having an amino acid sequence unique to hepatitis virus type 4,5 or 6; but excluding core sequences of type 4.

14. A peptide according to claim 13 which includes an antigenic region.

15. A peptide according to claim 14 from the NS4 region or antigenic part thereof.

16. A peptide according to claim 15 from the NS4 region of HCV-4 or an antigenic part thereof.

17. A peptide according to claim 15 from the NS4 region of HCV-5 or an antigenic part thereof.

18. A peptide according to claim 15 from the NS4 region of HCV-6 or an antigenic part thereof.

19. A peptide according to claim 14 from the NS5 region or an antigenic part thereof.

20. A peptide according to claim 19 from the NS5 region of HCV-4 or an antigenic part thereof.

21. A peptide according to claim 19 from the NS5 region

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of HCV-6 or an antigenic part thereof.

22. A peptide according to claim 14 having an antigenic sequence selected from the following:

HCV type 4 QPAVIPDREVLVYQQFEDEM

HCV type 4 ECSKHLPLVEHGLQLAEQF

HCV type 5 RPAIIPDREVLVYQQFDEM

HCV type 5 ECSTSLPYMDEARAIAGQF

HCV type 6 KPAVVPDREILVYQQFDEM

HCV type 6 ECSRHIPYLAEGQQIAEQF

and sequence variations thereof having deletions, insertions or substitutions which continue to provide an antigenic peptide.

23. A peptide according to claim 22 which is bound to a multiple antigen peptide core.

24. A peptide according to claim 23 having a sequence selected from the following:

HCV type 4 [H<sub>2</sub>N-QPAVIPDREVLVYQQFEDEM]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

HCV type 4 [H<sub>2</sub>N-ECSKHLPLVEHGLQLAEQF]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

HCV type 5 [H<sub>2</sub>N-RPAIIPDREVLVYQQFDEM]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

HCV type 5 [H<sub>2</sub>N-ECSTSLPYMDEARAIAGQF]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

HCV type 6 [H<sub>2</sub>N-KPAVVPDREILVYQQFDEM]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

HCV type 6 [H<sub>2</sub>N-ECSRHIPYLAEGQQIAEQF]<sub>8</sub>K<sub>4</sub>K<sub>2</sub>K-COOH

where K<sub>4</sub>K<sub>2</sub>K is the multiple antigen peptide core.

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25. A peptide according to any of claims 13 to 22 which is fused to another peptide to form a fusion peptide.

26. A peptide according to claim 25 fused to another peptide selected from the group consisting of  $\beta$ -galactosidase, glutathione-S-transferase, trpE or polyhedrin coding sequence.

27. A peptide according to any of claims 13 to 26 which is labelled.

28. An antibody to an antigenic peptide of any of claims 14 to 27.

29. A vaccine formulation which comprises an antigenic peptide of any of claims 14 to 27.

30. An immunoassay device which comprises a solid substrate having attached thereto an antigenic peptide according to any of claims 14 to 27.

31. A device according to claim 30 for HCV typing which comprises a plate having a series of locations respectively containing HCV-4, HCV-5 and HCV-6 antigenic peptides.

32. A device according to claim 31 wherein each location includes a mixture of antigenic blocking HCV peptides, the

- 61 -

mixture in each location excluding the peptide of the HCV type which that location is intended to detect.

33. A device according to claim 31 or 32 for the detection of HCV types 1 to 6.

34. An immunoassay device which comprises a solid substrate having attached thereto an antibody according to claim 28.

35. A method of in vitro screening a sample for HCV antibodies which comprises:

carrying out an immunoassay employing an antigenic peptide according to any of claims 14 to 27; and detecting any antibody-antigen complex produced.

36. A method according to claim 35 wherein the antigenic peptide is attached to a substrate and any HCV antibody present in the sample becomes bound to the peptide.

37. A method according to claim 36 wherein a mixture of antigenic blocking HCV peptides is applied to the antigenic peptide attached to the substrate together with the sample, the mixture excluding said attached antigenic peptide of the HCV type which is intended to be detected.

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38. A method according to claim 37 wherein a said mixture of antigenic peptides including said attached antigenic peptide is attached to the substrate.

39. A method according to claim 35 wherein any HCV antibodies present in the sample are captured on the substrate, and then said antigenic peptide is applied thereto for detection of any captured HCV antibodies.

40. A method of in vitro testing of a sample for HCV which comprises reverse transcribing any HCV polynucleotide present in the sample, amplifying by polymerase chain reaction, and detecting the amplified HCV polynucleotide by employing as a probe a polynucleotide according to any of claims 1 to 12.

41. A method of in vitro HCV typing which comprises carrying out endonuclease digestion of an HCV-containing sample to provide restriction fragments, the restriction pattern being characteristic of HCV-4, HCV-5 or HCV-6.



# FIG. 1

NS5 REGION

2659

2695

1a 12 IYQCCDLDPQARV<sub>10</sub>A<sub>1</sub>K<sub>10</sub>SLTERLYVGGPLTNSRGCCG

T<sub>1</sub> R<sub>2</sub>  
M<sub>1</sub>

4a 3 V . . . N<sub>1</sub> E<sub>2</sub> E . . K V<sub>2</sub> T<sub>2</sub> A . . . 2'2'2' . . . MH . . . K . DL . .

S<sub>1</sub> L<sub>1</sub> . . A<sub>1</sub>  
H<sub>1</sub>N<sub>1</sub>E<sub>1</sub>

6a 1 . . . S . Q . . . V . . R . VS . . . . . MV . . . K . QS . .

2696

2732

1a 12 YRRCRASG<sub>11</sub>VLT<sub>11</sub>TTSCGNTLT<sub>11</sub>TC<sub>11</sub>YIKAR<sub>9</sub>ACRAAG<sub>11</sub>L<sub>11</sub>Q<sub>8</sub>DCCT

R<sub>1</sub> I<sub>1</sub> R<sub>1</sub> Q<sub>3</sub>  
V<sub>1</sub> R<sub>4</sub>

4a 3 . . . . . F<sub>1</sub> . . . F . . . . . L . . . T<sub>2</sub> . . I . . . . . R<sub>2</sub> . . 2' .

Y<sub>1</sub> D<sub>1</sub>

6a 2 . . . . . P . . M . . . I . . . L . . H . . . . . NI K . . D

FIG. 2

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Type 4NS5 REGION

EG7	GTCTATCAGTGTGTGACCTGGAGCCCGAAGCCCGCAAGGTTATTGCTGCCCTCACAGAAAGACACAAT
EG13	GTCTATCAGTGTGTAACTGGAGCCCGAAGCTCGCAAGGCTATTACTGCCCTCACAGAAAGACTCTAC
EG19	GTCTATCAGTGTGTAGTCTGGAGCTCGAGGCTCGCAAGGTTATTACTGCCCTCACGGAAAGACTCTAC
EG7	GAGGGCGGCCCATGCACAACAGCAA.....
EG13	GTGGGCGGCCCATGCACAACAGCAAGGGAGACCTTTGTGGGTATCGGAGATGTGGGGCAAGCGGAGTC
EG19	GTGGGCGGCCCATGCACAATAGCAAGGGAGACCTTTGTGGGTACCGGAGATGCCGGGCAAGCGGAGTC
EG7	.....CCACACTGACGTGCTATCTCAAAGCCGACGCGCTATCAGAGCGGCAGGC
EG13	TTTACGACCAAGCTTCGGAAACACGCTGACGTGCTACCTAAAAGCCACGGCCGCTATTAGAGCGGCGGGG
EG19	TATACGACCAAGCTTCGGAAACACGCTGACGTGCTACCTCAAAGCCACAGCCGCTATTAGGGCGGCGGGA
EG7	CTGAGAGACTGCACC
EG13	CTGAGAGACTGCACT
EG19	CTAAAAGACAGCACT

Type 6

HK2	ATCTATCAGTCTTGCCAGCTGGATCCCGTAGCAAGGAGGGCAGTATCATCCCTGACAGAACGGCTCTAC
HK2	GTAGGCGGCCCATGGTGAACCTCAAGGGACAGTCATGTGGCTACCGTAGATGCCGCGCCAGTGGGGTG
HK2	CTGCCCACGAGCATGGGAAACACCATCACGTGCTATCTGAAGGCACACGCC---TGCAGGGCGGCCAAC
HK2	ATCAAGGACTGTGAC

Sequences shown correspond to positions 7975 to 8196 in Choo *et al.*, (1991)

"." : Sequence not determined

"-" : Gap introduced into sequence to preserve alignment

**FIG. 3**

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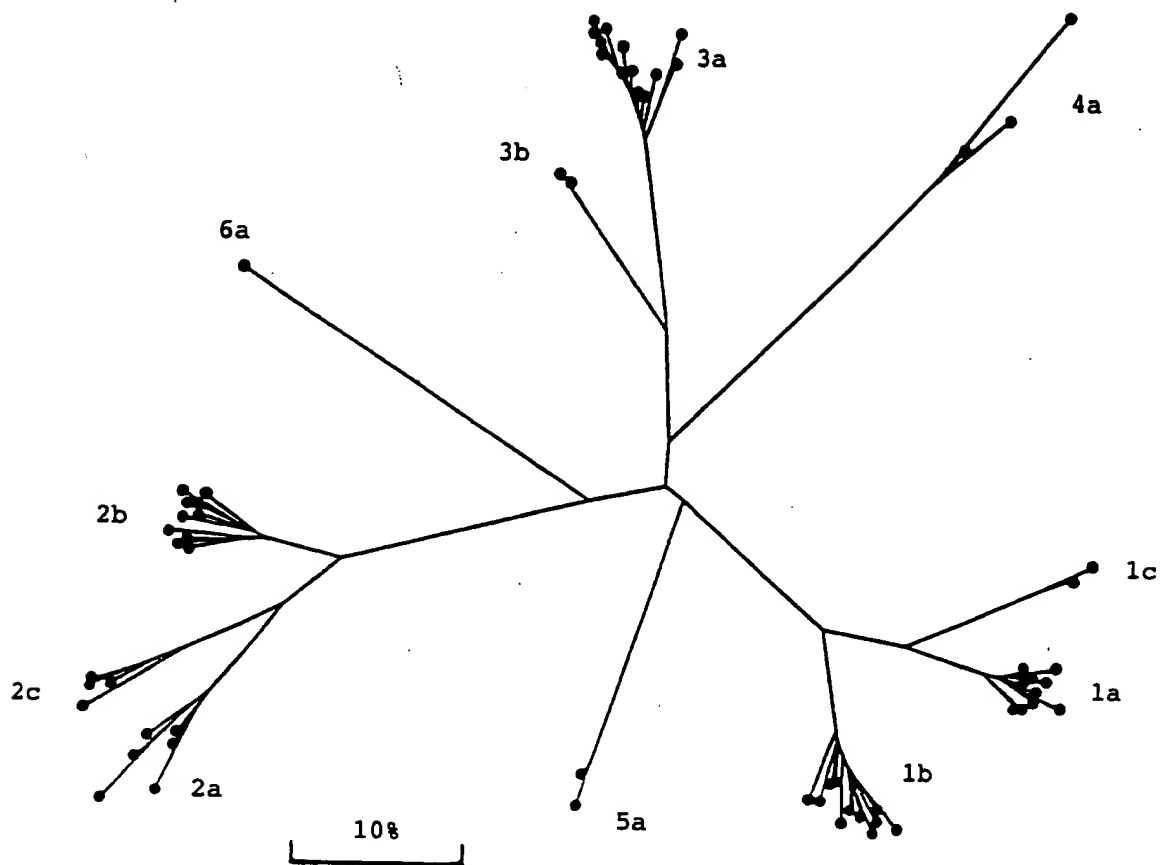


FIG. 4

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PCR primers Legend: +, sense; -, antisense; O, outer; I, inner.

007 AACTCGAGTATCCCACTGATGAAGTTCCACAT O -

435 CACCCATCACCAAATACAT O +

5351 TTTTGGATCCATGCATGTCAGCTGATCTGG I -

5943 TTTTGGATCCACATGTGCTTCGCCCAGAA I -

Primers 253, 281 and 221 are described in Simmonds *et al.*, 1993, *J. Clin. Microb.* 31: 1493.

FIG. 5

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TYPE

NS4 REGION 1

1	AAGCCGGCT	GTC/ATT	ATTCCCGACAGGGAAGTTCTCTACCAGGAGTTCGATGAAATG	54
	K P A	V / I	I P D R E V L Y Q E P D E M	18
2	CGAGTGGTC	GTGA	CTCCGGACAGGAGGTCCTCTATGAGGCTTTTGACGAGATG	54
	R V V	V	T P D K E V L Y E A P D E M	18
3	AAGCCGGCA	TTG	GTTCCAGACAAAGAGGTGTGTATCAACAATACGATGAGATG	54
	K P A	L	V P D K E V L Y Q Q Y D E M	18
4	CAGCCTGCT	GTT	ATCCCTGACCCGAGGTGCTCTACCAGCAGTTCGACGAAATG	54
	Q P A	V	I P D R E V L Y Q Q F D E M	18
5	AGACCTGCC	ATC	ATCCCGATAGAGAGGTGTGTACCAGCAATTTGATAAGATG	54
	R P A	I	I P D R E V L Y Q Q F D K M	18
6	AAGCCTGCT	GTT	GTCCCTGATCGCGAGATCTTATACCAGCAGTTTGACGAGATG	54
	K P A	V	V P D R E I L Y Q Q F D E M	18

TYPE

NS4 REGION 2

1	GAGTGGCCTCACACCTCCCTTACATCGAGCAGGGA	ATG/GCC	CAGCTCGCCGAG	CAATTC
	E C A S H L P Y I E Q G	M / A	Q L A E	Q F
2	GAATGTGCCTCTAGAGCGGCCCTCATTGAAGAGGGG	CAG	CGGATAGCCGAG	ATGCTG
	E C A S R A A L I E E G	Q	R I A E	M L
3	GAGTGCTCGCAAGCTGCCCATATATCGAACAAGCT	CAG	GTGATAGCCAC	CAGTTC
	E C S Q A A P Y I E Q A	Q	V I A H	Q F
4	GAGTGTCCAAACACCTTCCACTAGTCGAGCACGGG	TTG	CAACTTGTGAG	CAATTC
	E C S K H L P L V E H G	L	Q L A E	Q F
5	GAGTGCTCGACCTCGCTCCCTATATGGACGAGGCA	CGT	GCTATTGCCGGG	CAATTC
	E C S T S L P Y M D E A	R	A I A G	Q F
6	GAGTGCTCTAGGCACATCCCCTACCTCGCTGAGGGC	CAG	CAGATCGCCGAA	CAGTTC
	E C S R H I P Y L A E G	Q	Q I A E	Q F

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/00957

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5 C12N15/51 C12N15/40 A61K39/29 C12P21/08 C12N15/62  
G01N33/576 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N A61K C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF GENERAL VIROLOGY, vol.74, no.4, April 1993, READING, UK pages 661 - 668 P.SIMMONDS ET AL. 'Sequence variability in the 5' non-coding region of hepatitis C virus: identification of a new virus type and restrictions on sequence diversity' cited in the application see page 667, right column --- -/--	1-41

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- \*E\* earlier document but published on or after the international filing date
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Date of the actual completion of the international search

19 September 1994

Date of mailing of the international search report

13. 10. 94

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/00957

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF GENERAL VIROLOGY, vol.73, no.5, May 1992, READING, UK pages 1131 - 1141 S.-W. CHAN ET AL. 'Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants' cited in the application see page 1133, last paragraph - page 1135; figure 3	1-41
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.89, no.15, 1 August 1992, WASHINGTON US pages 7144 - 7148 T.-A. CHA ET AL. 'At least five related, but distinct, hepatitis C viral genotypes exist' cited in the application see page 7148, left column	1-41
P,X	THE JOURNAL OF GENERAL VIROLOGY, vol.74, no.11, November 1993, READING, UK pages 2391 - 2399 P.SIMMONDS ET AL. 'Classification of hepatitis C virus in six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region' see figure 3	1-41